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Host controls of HIV broadly neutralizing antibody development

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Summary

Induction of broadly neutralizing antibodies (bNAbs) is a major goal of HIV vaccine development. BNABs are made during HIV infection by a subset of individuals but currently cannot be induced in the setting of vaccination. Considerable progress has been made recently in understanding host immunologic controls of bNAb induction and maturation in the setting of HIV infection, and point to key roles for both central and peripheral immunologic tolerance mechanisms in limiting bNAb development. Immune tolerance checkpoint inhibition has been transformative in promotion of anti-tumor CD8 T-cell responses in the treatment of certain malignancies. Here, we review the evidence for host controls of bNAb responses, and discuss strategies for the transient modulation of immune responses with vaccines toward the goal of enhancing germinal center B-cell responses to favor bNAb B-cell lineages and to foster their maturation to full neutralization potency.

Keywords

AIDS; autoantibodies; autoimmunity; B cells; vaccination

1 | INTRODUCTION

A major conundrum in HIV-1 vaccine development work is why highly antigenic vaccine envelopes (Envs) do not induce broadly neutralizing antibodies (bNAbs) when used as vaccine immunogens. Various HIV-1 envelope glycoprotein (Env) forms exist that are bound by both mature bNAbs and their unmutated common ancestor antibodies (UCAs). To date, however, even though Env designs and transmitted/ founder Envs that initiate bNAb lineages by UCA engagement exist (Bonsignori and Haynes, this volume; Stamatatos, this volume 275), no HIV-1 vaccine regimen has been designed to date that induces the full maturation of bNAb lineages.

Shortly after the isolation of the first bNAbs it was noted that these antibodies had one or more unusual traits, including high frequencies of V(D)J mutations, significantly extended third complementarity determining regions in the heavy-chain variable region (HCDR3), and

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CONFLICTS OF INTEREST

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poly-and/or autoreactivity with human lipids and proteins. These findings prompted the hypothesis that the generation of bNAbs was disfavored by immune tolerance mechanisms.^{1,2} Initial support for this hypothesis came from the demonstration that three bNAbs, including 2F5 and 4E10, avidly bound to human autoantigens.¹ The high frequencies of V(D)J mutation in many bNAbs—as high as 30%—could also be explained as the consequence of tortuous somatic evolution to avoid tolerization while maintaining the capacity to neutralize HIV-1.³

Two strategies were developed to test the hypothesis that failure to generate HIV-1 bNAbs was, at least in part, the result of HIV-1 mimicry of host determinants and the consequent purging by immune tolerance of those B cells best able to bind the shared host/HIV-1 epitopes.³ First, V(D)J knockin (KI) mice expressing bNAb BCR were generated to determine whether the auto-/polyreactivity of bNAb BCR determined in vitro was sufficient to activate immune tolerance as determined by clonal deletion, receptor editing, and/or B-cell anergy (Table 1). Second, the bNAbs were used to identify and isolate host molecules that were structurally mimicked by neutralizing epitopes of HIV-1 Env. Failure in either test would have disproven the “immune tolerance” hypothesis for the lack of protective humoral immunity to HIV-1.

2 | EVIDENCE FOR HOST CONTROL OF BROADLY REACTIVE NEUTRALIZING ANTIBODIES

2.1 | bNAb expression by knockin mouse lines

Haynes et al.¹ first demonstrated that the gp41 membrane proximal external region (MPER) bNAbs, 2F5, and 4E10, were polyreactive for human host lipids and several proteins. In 2010, Verkoczy et al.⁴ demonstrated that 2F5 bNAb V_HDJ_H knockin (KI) mice exhibited profound deletion (95%) of maturing B cells in bone marrow and that the great majority of B cells in the periphery were anergic. Since then, a number of bNAb KI mouse models have been developed and studied both in the presence and absence of HIV-1 vaccine immunization (Table 1; Verkoczy, Tian, and Alt, this volume).

2.2 | gp41 MPER bNAbs

We have previously shown that the 2F5 bNAb that binds to the proximal MPER of HIV-1 Env, has reactivity for lipids, and avidly binds to an ELDKWA amino acid epitope present in both gp41 Env and an enzyme of tryptophan metabolism, kynureninase (Kynu).⁵ KI mice expressing the V_HDJ_H+V_LJ_L rearrangements of the gp41 MPER bNAbs 2F5 and 4E10, exhibit severe defects in B-cell development; in both lines, >95% of immature and transitional B cells are lost at the first tolerance checkpoint and the great majority of peripheral B cells exhibit anergic phenotypes.⁶ Double KI mice that express the germline (unmutated common ancestor, UCA) V_HDJ_H and V_LJ_L of the 2F5 bNAb (UCA 2F5 KI) exhibit an even more profound central deletion of developing B cells and peripheral anergy.⁷ Immunization of 2F5 bNAb KI mice with an MPER peptide-liposome designed to mimic MPER epitopes as expressed on virions in conjunction with a TLR-4 agonist (monophosphoryl lipid A) could reverse the anergic state of peripheral B cells, allowing 2F5 KI mice to produce 2F5 bNAb.⁸ Immunization of UCA 2F5 KI mice with the same

immunogen and adjuvant resulted in activation of peripheral B cells expressing the 2F5 UCA B cell receptor (BCR), but failed to induce immunoglobulin class switching (Ig CSR) or somatic hypermutation (SHM).⁸ Similarly, in rhesus macaques immunized with the MPER peptide-liposome, B cells expressing orthologous VDJ rearrangements of the human 2F5 V_HDJ_H were initially activated, but rapidly disappeared following additional immunizations.⁷ This immunization protocol did induce serum antibody responses to the ELDKWA site in Kynu, presumably by breaking tolerance to this phylogenetically conserved epitope.^{5,9} Moreover, the immunized macaques that mounted Kynu-specific antibody responses also generated MPER-reactive antibodies that bound the 2F5 gp41 epitope; these gp41 MPER antibodies were limited, however, in neutralization potency by a limiting ceiling of HCDR3 hydrophobicity obtainable by vaccination-induced somatic mutation that is needed for 2F5 and 2F5-like antibodies to react with virion lipids to effectively neutralize HIV-1.⁷

Similar to bNAbs 2F5, the 4E10 MPER bNAbs exhibit broad polyreactivity with lipids and host proteins and a high degree of autoreactivity for an RNA splicing factor, splicing factor 3b subunit 3 (SF3B3).^{1,5,9} Indeed, KI mouse lines expressing the 4E10 V_HDJ_H or 4E10 V_HDJ_H + V_LJ_L rearrangements exhibited high levels of B-cell deletion at the first checkpoint and peripheral B cells that express anergic phenotypes; the majority of peripheral, mature B cells appear to have escaped central tolerance by receptor editing.^{6,10} Moreover, in vitro, the 4E10 bNAbs had blocking effects on clotting tests as well as lupus anticoagulant activity and when infused into humans, prolonged both the plasma partial thromboplastin time and prothrombin time in vivo.^{1,11} These effects demonstrate the physiological significance of 4E10 polyreactivity.

To date, 4E10 is the only HIV-1 bNAbs demonstrated in vitro and in vivo to have a potentially negative, off-target, physiologic function.^{1,11} Interestingly, the lipid reactivity of the 4E10 bNAbs is substantially greater than that of 2F5,^{1,5} and induction of antibodies to the 4E10 distal MPER epitope has proved more difficult than induction of antibodies to the proximal MPER 2F5 epitope.⁷ In contrast, we recently completed a study of the induction of 2F5 antibody responses in 2F5 KI mice and 2F5-like, Kynu-reactive antibodies in Rhesus macaques that demonstrated that neither 2F5 nor 2F5-like antibodies from the blood inhibit Kynu enzyme activity or perturb tryptophan metabolism in blood or brain; thus, the inherent autoreactivity of the 2F5 bNAbs does not induce tissue damage in vivo (Bradley, T, Kelsoe, G and Haynes, BF, J Immunol 2016, in press).

2.3 | CD4 binding site bNAbs

CD4 binding site (CD4-bs) bNAbs can be divided into two major groups based on how the antibody paratope interacts with the CD4-bs epitope. CD4-bs bNAbs that bind primarily via contacts in the third complementarity determining region of the heavy chain (HCDR3) include the CH103, HJ16, VRC13, and VRC16 antibodies; a second group, including the VRC01 and 3BNC60, ANC131, and CH235 bNAbs bind largely via contact residues in HCDR2, the second H-chain CDR.¹²

2.4 | VRC01-class bnAbs

The prototype VRC01 antibody is not polyreactive against human proteins but does have high avidity for the phylogenetically conserved ubiquitin protein ligase E3A (UBE3A).⁹ VRC01 UCA KI mice with the HCDR3 of the UCA mutated are not deleted in bone marrow, but immunization of these mice with Envs designed for VRC01 UCA binding induces limited somatic mutations or affinity maturation. Recently, Tian and Alt¹³ have described a KI mouse line with the V_HJ_H and the V_LJ_L of VRC01 UCA expressed in a pre-rearranged format that allows large numbers of distinct V(D)J rearrangements to be generated. Repeated immunizations of these KI mice with multimerized Envs designed to be bound by the VRC01 UCA,^{14,15} Tian, and Alt demonstrated induction of substantially mutated VRC01-like B cells and neutralizing serum antibody capable of neutralizing tier 2 HIV-1 isolates that had lost glycans decorating the periphery of the CD4-bs (Cell, in press). Despite this progress toward bnAb activity, however, after five immunizations, the elicited VRC01-like antibodies remained unable to neutralize wild-type HIV-1 strains that expressed Env glycoproteins with glycan residues adjacent to the CD4-bs (Tian and Alt Cell, in press). If this block in bnAb development is the consequence of tolerization—perhaps the result of UBE3A autoreactivity—it becomes manifest only late in the development of the VRC01-class lineage. This late immune control may reflect negative selection in the germinal center microenvironment.^{16–18}

In contrast to the VRC01 KI line generated by Tian et al.¹³, McGuire et al.¹⁹ have reported extensive B-cell deletion in the bone marrow of KI mice bearing the UCA 3BNC60 V(D)J rearrangements, high frequencies of peripheral B cells with anergic phenotypes, and evidence for extensive receptor editing by secondary rearrangements of the light-chain locus. Interestingly, whereas a soluble, trimeric form of Env immunogen was unable to induce proliferation in peripheral, anergic B cells in UCA 3BNC60 KI mice, higher order multimers could, suggesting that anergic B cells may be activated by some immunogen forms.¹⁹ In fact, particulate antigens are known to be capable of bypassing the anergic state in other experimental models.^{20,21}

It may be noteworthy that while all VRC01-class antibodies require the HCDR2 of the V_H1-2*02 gene segment to be paired with a light-chain VJ rearrangement containing a LCDR3 of five amino acid residues, the UCA VRC01 KI mice generated by Tian et al.¹³ utilized V κ 3-20, while in the UCA 3BNC60 line generated by McGuire et al.¹⁹, the V_H1-2*02 rearrangement was paired with V κ 1-33*01. Comparison of these two KI lines, UCA VRC01 with normal B-cell development and UCA 3BNC60 with strong tolerization at the first checkpoint, suggests that the light chain variable regions may determine the developmental/differentiative fates of the VRC01-class of bnAbs (Tian and Alt Cell, in press).

2.5 | CH103-class bnAbs

The CH103 bnAb binds to the CD4-bs via residues in HCDR3 and in its original description it, but not the UCA CH103 was observed to be polyreactive in clinical autoantibody assays.²² A subsequent study using human protein microarrays⁹ also found the mutated, mature form of CH103 to be significantly polyreactive and, like three other CD4-bs bnAbs

(VRC01, VRC02, and CH106) react with UBE3A with avidities proportional to their neutralization potency (Figure 1). This association raises the possibility that CD4-bs bnAbs of both the HCDR3 and HCDR2 classes may be controlled by central and/or peripheral (eg, germinal center) immune tolerance acting to suppress autoreactivity to UBE3A.

2.6 | V1V2-glycan bnAbs

The V1V2 bnAbs are generally characterized by exceptionally long HCDR3 domains: the CH01 V1V2 bnAb HCDR3 contains 24 amino acid residues,²³ the PG9 and PG16 bnAbs have HCDR3s of 28 residues,²⁴ and bnAb -VRC26 has an extremely long HCDR3 of 35 amino acids.²⁵ These contrast substantially to the 15 residue mean value for human HCDR3 lengths. Of four CH01 V1V2-glycan bnAbs isolated, CH03 reacted with histones, ribonucleoprotein, and centromere autoantigens, while CH01, CH02, and CH03 bound to the hepatitis C E2 glycoprotein and to whole cell lysates of intestinal flora.²⁶ V1V2 bnAbs CAP256-VRC26, PG9 and PG16 were not described to be polyreactive²⁴ but B cell antigen-receptors with long HCDR3 regions are commonly culled from the primary B-cell repertoire at the first immune tolerance checkpoint.^{27–29} Thus, V1V2-glycan antibodies with very long HCDR3s may be limited by deletion in bone marrow, and polyreactivity of more mature antibodies may limit their maturation at the later stages of affinity maturation.

2.7 | V3-glycan bnAbs

Relatively little is known with regard to the auto- or polyreactivity of V3-glycan bnAbs or their ability to affect B-cell development and maturation. KI mice with the DH270 V3-glycan lineage are under construction (Tian, M and Alt, F unpublished), but to date, a limited number of studies on V3-glycan UCAs and affinity matured bnAbs have not demonstrated polyreactivity. V3-glycan bnAbs, however, often carry long HCDR3 motifs; for example, the PGT121 bnAb has a long HCDR3 and is capable of inhibiting CD4 binding to Env gp120 even though it does not bind to the CD4-bs.³⁰ It is thought that PGT121 binding induces an allosteric change in the HIV-1 Env that minimizes or precludes CD4 binding.³⁰

Thus, accumulating data indicate that most bnAb lineages, at some point in their evolution, are limited by various types of immune tolerance controls, including deletion, receptor editing, anergy, or affinity reversion/antibody redemption by hypermutation.^{8,21} The generation of many bnAbs to the HIV-1 Env is restricted by host epitope mimicry as evidenced by deletion of bnAb clones, either during B-cell development or in germinal centers (GC) is a significant component for sub-optimal immune responses to current HIV-1 Env vaccines. Substantial evidence indicates that this physiologic process of B-cell elimination applies to the germline predecessors of the production of bnAbs targeting the membrane proximal external region of gp41 (MPER, eg, 2F5 or 4E10) and the anti-CD4 binding site on gp120 (CD4bs, eg, VRC01, CH103). As noted above, elimination of B cells expressing the 2F5 or 4E10 (both germline and mutated) receptors by immune tolerance has been demonstrated in KI mice^{4,8,10,31}; for these epitopes, HIV-1 mimicry of host antigens is no longer a hypothesis. We think it likely that for the induction of robust bnAb activity in the setting of vaccination, vaccine formulations and adjuvants will need to be designed to transiently overcome tolerance controls and provided in a sequence that promotes normally

disfavored pathways of clonal selection and complete development of bNAb B cell lineages.^{32,33}

2.8 | Identification of human proteins mimicked by HIV-1

Knowing that the 2F5 and 4E10 KI mice exhibited patterns of B-cell deletion and anergy consistent with the action of immune tolerance,^{4,6,10} Yang et al.⁵ used the bNAbs themselves to search for murine proteins that were structurally similar to the HIV-1 Env epitopes recognized by each antibody by immunoprecipitation and mass spectroscopy. In this way, we identified 15 cytoplasmic proteins reproducibly bound 2F5 and three proteins recognized by 4E10.⁵ In an independent screen for 2F5 and 4E10 ligands, we used a commercial protein microarray that covers 9400 human proteins, to identify any binding by the 2F5 and 4E10 bNAbs to human proteins; remarkably, the overlap between immunoprecipitation of murine proteins and binding to arrayed human proteins was very substantial. In this way, we identified 11 human proteins that in soluble form were bound by the 2F5 or 4E10 bNAbs. Under more stringent binding conditions,³⁴ the numbers of host targets could be further reduced to two proteins [Kynu and CMTM3 (CKLF-like MARVEL transmembrane domain containing 3)] for 2F5 and a single protein [SF3B3 (splicing factor 3b subunit 3)] for 4E10.⁵

Serological testing, western blotting, and co-localization by fluorescence microscopy demonstrated that the 2F5 and 4E10 bNAbs recognized the same proteins bound by antibodies to Kynu or SF3B3.⁵ Collectively, these data strongly indicated that Kynu and SF3B3, proteins that are phylogenetically conserved in mice, non-human primates, and humans are the primary self-antigens of 2F5 and 4E10.

With regard to Kynu autoreactivity by bNAb 2F5, HIV-1 mimicry was virtually perfect. The nominal 2F5 epitope, a linear epitope specified by the Glu₆₆₂-Leu-Asp-Lys-Trp-Ala₆₆₇(ELDKWA) residues of the HIV-1 gp41 MPER ectodomain³⁵ is matched exactly in the Kynu H4 domain α -helix; remarkably, Kynu is the only known protein besides HIV Env to contain the complete 2F5 epitope motif.⁵ Immunization of opossums, the only mammal known not to share the MPER/Kynu epitope (ELEKWA), resulted in MPER-specific antibody responses that were 1000-fold greater than those of mice, rats, and guinea pigs [⁵ and unpublished data]. In contrast, opossum Ab responses to the adjacent 4E10 epitope were minimal or absent, consistent with the presence of the highly conserved MPER/SF3B3 epitope in opossums.⁵

Subsequently,⁹ we characterized off-target binding in 22 bNAbs from 14 distinct clonal lineages, covering four major neutralization sites for HIV-1³⁶: the VRC01 family (VRC01, VRC02, VRC03, VRC07, and NIH45046), CH98 (CH98), CH30-34 (CH31), and CH103-106 (CH103, CH106) lineages recognize the CD4bs; 2F5, 4E10, 10E8, and CH12 lineages bind the MPER; PG9/PG16 (PG9, PG16), CH01-04 (CH01, CH03), and PGT141-145 (PGT145) lineages map to the V1V2-glycan epitopes; and 2G12, PGT121-123 (PGT121), and PGT125-131 (PGT125, PGT128) lineage recognizes V3-glycans. In comparison, we tested nine independent, non-neutralizing or autologous neutralizing (ie, strain-specific) HIV-1 Abs (nNAbs) specific for HIV-1 Env V2 and V3 loop epitopes, and CD4-induced (CD4i) gp120 epitopes. The inclusion of NNABs was important as it remained possible that the unusual characteristics of bNAbs was not a property of their paratopic

specificities but rather of development in the milieu chronic inflammation and immune dysregulation characteristic of chronic HIV-1 infections.⁹

This study found that 45% (10/22) bNAbs (VRC07, NIH45-46, CH103, CH106, CH98, CH31, 4E10, PG9, PGT125, and PGT128) were significantly polyreactive for human proteins and at least one polyreactive bNAb was present in each of the four neutralization classes: CD4bs, MPER, V1/V2, and V3-glycan.^{5,9} In contrast, only a single (1/9; 11%) nNAb (HG131) recovered from a vaccinee (RV144 ALVAC prime, AIDSVAX B/E protein boost) was found to be polyreactive. The different frequencies of polyreactivity among bNAbs and nNAbs was highly significant whether analyzed by lineages or single Abs.⁹

Of the 12 non-polyreactive bNAbs, three [VRC01, 10E8, and 2F5; (3/12; 25%)] reacted strongly⁵ with specific human proteins: (VRC01) ubiquitin-protein ligase 3A (UBE3A); (10E8) family with sequence similarity 84, member A (FAM84A); and (2F5) kynureninase (KYNU) and CKLF-like MARVEL transmembrane domain containing 3 (CMTM3).⁵ Only a single nNAb (1/8; 13%) strongly bound to a human protein: (19b) parkin coregulated gene protein homolog (PACRG).

The different frequencies of poly- or autoreactive bNAbs (59%) vs nNAbs (22%) was significant and uncorrelated with mutation frequencies per se.⁹ Importantly, four CD4bs bNAbs, VRC01, VRC02, and CH106 and CH103, bound human UBE3A more avidly than the control Ab (Figure 1). Although VRC01 alone bound UBE3A 500-fold above the 151K control, VRC02 and CH106 bound UBE3A 300-fold more avidly while CH103 bound UBE3A $\approx$ 100-fold more avidly than 151K. Binding to UBE3A was confirmed for all four bNAbs in stringent condition ELISA (Figure 1B).⁵ Surface plasmon resonance estimates of UBE3A:VRC01 binding strength suggested a $K_d \approx 30$ nM, an avidity within the range of autoreactive human Abs derived from autoimmune patients.³⁷

Demonstration that the MPER bNAbs 2F5 and 4E10 are controlled by immunological tolerance,^{4-6,10,31} the identification of human protein epitopes that are avidly bound by specific bNAbs,⁵ and our identification of UBE3A as a likely self-antigen recognized by HIV-1 bNAbs⁹ provide strong and direct evidence of host mimicry by HIV-1. Such mimicry conceals vulnerable neutralization sites by hiding in plain sight: immunological tolerance purges the primary immune response of those lymphocytes most suited for protective immunity and/or removes those B cells that acquire bNAb specificities during somatic evolution. Although this method of immune evasion can be highly effective,^{5,38} we think that it is not broadly appreciated. We hypothesize that host mimicry by pathogens is evolutionarily advantageous and significantly more widespread than generally believed.³⁹

Over the past 10 years, many novel HIV-1 bNAbs have been isolated, and to date, all carry one or more of the unusual, but characteristic, traits that are the hallmarks of bNAbs: long HCDR3s, extensive V(D)J hypermutation, and poly- or autoreactivity.⁴⁰ A number of independent studies have demonstrated the control of various types of bNAbs by either central or peripheral host immune tolerance controls in bNAb KI mouse lines (Table 1). Moreover, we and others have demonstrated that as a group, HIV-1-infected individuals who make bNAbs have significantly higher frequencies of plasma autoantibody, increased

numbers of T follicular helper cells circulating in blood and lower numbers of T regulatory cells, and higher expression of PD-1 on T regulatory cells that is associated with impaired regulatory capacity than do matched patients that do not make bNAbs.³³ These correlations support the idea that the ability to make bNAbs is positively associated with enhanced humoral responses and the relaxation of central and/or peripheral tolerance checkpoints. Thus, considerable data are emerging for the role of immune tolerance mechanisms in controlling bNAbs, raising the possibility of induction of bnAbs by recreating those viral events and immune perturbations that transpire in the setting of HIV infection with an immunogen formulation in the setting of vaccination.³³

3 | ENHANCING IMMUNITY WITH CHECKPOINT INHIBITION THERAPY

Methods to enhance immune responses and to suppress the physiological checkpoints that control the intensity and specificity of immunity are available. Immune checkpoint therapy targets regulatory pathways that regulate T-cell proliferation and function to enhance immune responses to malignancies. This relatively new therapeutic approach has resulted in significant clinical advances, and in many patients has elicited long-term clinical benefits. Despite substantial ignorance of how and when physiological activating and inactivating signals cooperate to control immune responses, checkpoint inhibitor therapies represent a significant advance in tumor therapy and offer general insight into the physiologic regulators of immunity.^{41,42}

T cells are competent to respond to tumor cells expressing unique antigens, ie, retroviral antigens, cryptic differentiation antigens, and/or neoantigens generated by mutation as if the tumor is foreign. Tumor-specific antigens are processed and displayed by malignant cells and recognized by specific TCRs; this recognition drives a process of T-cell activation, proliferation, and differentiation that is limited by (i) the absence of co-stimulatory signals (CD80, CD86); (ii) engagement of inhibitory receptors that are expressed on activated T cells; and (iii) the activity of regulatory T cells.^{43–45}

3.1 | CTLA-4

The balanced activities of co-stimulatory and inhibitory receptors on T cells are key to immune checkpoint therapy, and the receptor ligand pairs that regulate T-cell activation or deactivation are the targets of therapeutic checkpoint inhibitors. The co-stimulatory T-cell receptor, CD28, its inhibitory counterpart CTLA-4, and their shared ligands, CD80 and CD86 (B7.1 and B7.2) are the best studied of co-signal pairs.^{41,42} In humans, CD28 and CTLA4 bind a third ligand, B7-H2, that can be induced in many tissue types and also serves as the ligand for another co-stimulatory receptor, inducible T-cell co-stimulator (ICOS).^{46,47}

T-cell inhibition by CTLA-4 is achieved through two mechanisms: competitive antagonism of CD28 signals and direct negative signaling (ref). Engagement of CD28 molecules by CD80 and CD86 in acute T-cell responses initiates a reorientation of intracellular CTLA-4 toward the site of TCR engagement, an increase in its surface expression, and clustering within the immunological synapse.⁴⁸ There, CTLA-4 competes with CD28 for CD80/CD86 engagement and because of its greater abundance and affinity for CD80/-86, reduces CD28-dependent co-stimulation available to the cell.

The second mechanism of CTLA-4-mediated T-cell inactivation is the delivery of inhibitory signals through its cytoplasmic tail, a mechanism inferred from observations that co-crosslinking of CTLA-4 and TCR in the presence of unlimited CD28 co-stimulation is still capable of inducing cell-cycle arrest and IL-2 downregulation.⁴⁸ In contrast to antagonism of CD28 co-stimulation, this mechanism is operational when CTLA-4 is expressed at low levels on the T-cell surface. The signals delivered by CTLA-4 and their mechanism of action remain uncertain; regardless of how the negative signals produced by CTLA-4 are propagated, CTLA-4 signaling downregulates cytokine production by inhibiting the accumulation of AP-1, NF- κ B, and NFAT in activated T cells and controls proliferation by inhibiting cyclin D3, cyclin-dependent kinases, and degrading p27^{kip1}.⁴⁸

3.2 | PD-1

A second inhibitory receptor-ligand pair targeted in checkpoint therapy is the programmed death-1 (PD-1) receptor (CD279) and its B7 ligands, PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273). This pathway acts in the event of persistent antigenic stimulation, including exposure to self-antigens, chronic viral infections, and tumors.^{41,45,49} The PD-1:PD-L pathway is the underlying cause of the 'T-cell exhaustion' associated with poor virus control during chronic infections.^{41,42,45}

The function of PD-1 is distinct from CTLA-4 in that PD-1 does not interfere with co-stimulation, but interferes with signaling mediated by the T-cell antigen receptor.⁵⁰ Unlike CTLA4, the cytoplasmic tail of PD1 contains an immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) that are phosphorylated following PD-1 ligation. PD-1 transduces an inhibitory signal when engaged simultaneously with the TCR or B-cell receptor but does not transduce a signal when crosslinked alone. Phosphorylation of the ITSM, recruits the SHP-2 (and smaller amounts of SHP-1) phosphatase to the PD-1 cytoplasmic domain. This recruitment leads to dephosphorylation of kinases activated by antigen-receptor signaling and reduces CD28-mediated activation of phosphatidylinositol-3-OH kinase, to suppress Akt phosphorylation, glucose metabolism, and expression of anti-apoptotic factors.⁵⁰ The amount of PD-1 expression and the extent of engagement of PD-1 by its ligands regulate the threshold for T-cell activation and quantities of cytokines produced.

The functional activity of co-signaling is more complex than simple enhancement or suppression of T-cell proliferation or function. For example, CTLA-4-B7 interaction mediates a bi-directional interaction that inhibits T-cell function directly but may also induce the expression of indoleamine 2,3-dioxygenase (IDO), which suppresses conventional T-cell activation but promotes the function of regulatory T cells.⁵¹ PD1 on conventional T cells is inhibitory, whereas on T_{Reg} cells, it enhances proliferation and survival.⁵²

That CTLA-4 and PD-1 regulate distinct inhibitory pathways and have non-overlapping mechanisms of action suggested that concurrent combination therapy with both might be more efficacious than either alone. Indeed, this prediction was demonstrated in mice and later supported by outcomes in human clinical trials.^{41,42} Combination treatments are also being developed to enable blockade of multiple inhibitory pathways, such as LAG-3^{53,54} and TIM-3^{55,56}; importantly checkpoint blockade has also been combined with cancer

vaccines^{57–59} so as to ‘fuel the engine (with vaccination) and release the brake (with checkpoint inhibitors)’.⁵⁹

3.3 | Regulatory T cells

Regulatory T (T_{Reg}) cells are a suppressive subset of $CD4^+$ T cells that function to antagonize immune responses. The development, persistence, and activity of T_{Reg} cells depends on the transcription factor forkhead box P3 (FoxP3) and identifies the various subsets of T_{Reg} cells from phenotypically similar, non-regulatory $CD4^+$ T cells. T_{Reg} cells are necessary to prevent damaging autoimmune responses and to mitigate the immunopathogenesis associated with some protective immune responses. Having too few T_{Reg} cells can trigger fatal autoimmunity [eg, IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome)], whereas having too many can result in the suppression of beneficial immune responses.

T_{Reg} cells constitute some 10% of peripheral $CD4^+$ T cells, are required for the maintenance of immune homeostasis. Humans with a debilitating mutation in the *FOXP3* gene develop IPEX syndrome, a severe, multi-organ autoimmune disease. Scurfy mice, which lack FOXP3 expression, also develop a lethal autoimmune syndrome. T_{Reg} cells are needed throughout life as prolonged depletion of FoxP3⁺ T_{Reg} cells in adult mice can result in a fatal autoimmunity within weeks.^{60,61}

T_{Reg} cells are classically defined by their constitutive expression of the IL-2 receptor α chain (CD25), CTLA-4, and the tumor necrosis factor (TNF)-receptor family members GITR (glucocorticoid-induced TNF receptor-related protein) and OX40.⁶² As these molecules are expressed by many lymphocytes, Foxp3 remains the definitive signature of T_{Reg} cells.⁴⁴ Despite extensive study, the mechanism by which T_{Reg} cells suppress immune responses in vivo is not well understood. T_{Reg} cells may inhibit responses indirectly by modulating antigen-presenting cell function,⁶³ directly by secreting anti-inflammatory cytokines,^{64,65} and/or by inducing production of indoleamine 2,3-dioxygenase (IDO), which degrades tryptophan, and consequently promotes T-cell quiescence and apoptosis.^{66,67} Just which suppressive mechanisms are employed by T_{Reg} cells is unclear, but in most, if not all cases, such mechanisms are likely redundant and may vary by tissue site or the degree of inflammation.⁶²

A recently described class of T_{Reg} cells is the follicular T_{Reg} (T_{FReg}) cell.^{68–70} As their name implies, T_{FReg} cells enter GCs and interact with resident follicular T-helper cells (T_{FH}) to regulate B-cell expansion, mutation, and V(D)J hypermutation. As the primary site of B-cell hypermutation and class-switch recombination, the GC response is well regulated to minimize generation of autoantibody, systemic autoimmune disease, pathological inflammation, and B-cell malignancy. Among these regulatory mechanisms, T_{FReg} cells specifically and potently suppress both T_{FH} and B cells in GCs.^{68–71} T_{FReg} cells are likely essential for the physiological homeostasis of GC reactions and may play a crucial role in determining GC persistence and, possibly, clonal selection. In the context of improved HIV-1 vaccines, T_{FReg} activity may be a controlling factor in the prevention of vaccine-induced bNAb development, either by limiting GC persistence to effectively reduce the acquisition of V(D)J mutations or by aiding in the removal of autoreactive mutant B cells.

Either activity would have significant consequences. For example, in bNAbs, V(D)J hypermutation is frequent not only in the antigen-contacting CDRs, but also in the antibody framework regions responsible for maintaining antibody structure; remarkably, these framework mutations may be necessary for bNAb breadth and potency.⁷² Somatic mutation can also generate de novo hotspots for activation-induced cytidine deaminase (AID) activity in V(D)J rearrangements, creating template sites prone to insertion/deletion mutations⁷³; in some cases, the occurrence of an insertional event is critical to acquisition of bNAb activity.⁷³ Thus, merely by limiting GC persistence, T_{FReg} activity might block the possibility vaccine-induced bNAb production.

4 | VACCINE TRANSIENT IMMUNE MODULATION FOR BNAB INDUCTION

The very properties that appear to be required for bNAb activity, extended HCDR3 regions, high frequencies of V(D)J mutation, poly- or autoreactivity are also properties that disfavor B-cell development and survival. Given that immunological tolerance shapes the primary BCR repertoire, only B cells that have been vetted by tolerance are available to respond to vaccines; for vaccine antigens that mimic self-antigens, the pool of mature B cells that can respond will be reduced.

These predicted effects have been amply demonstrated in bNAb KI mouse lines (Table 1) and are consistent with atypical pathways of bNAb evolution normally proscribed by the effects of peripheral tolerance.³ The unusually low efficiency with which immunization elicits bNAbs implies that bNAb B cells are the products of disfavored clonal evolution. In consequence, we propose that checkpoint inhibitors and other agents that transiently relax immunological tolerance may provide tools for increasing HIV-1 vaccine efficacy. Even should this approach not prove to be useful in large-scale vaccination efforts, they may point the way toward understanding why even our best vaccine antigens are unable to provide significant protection to at risk populations.

4.1 | First tolerance checkpoint

Immune tolerance purges developing B cells that express antigen receptors (BCR) that are self-reactive. At least three mechanisms for central B cell tolerance have been identified: apoptotic deletion, receptor editing, and functional inactivation or anergy.⁷⁴ Despite long study, the mechanisms that drive these tolerizing actions have not yet been made clear. It came, therefore, as a surprise when expression of activation-induced cytidine deaminase (AID) in immature and transitional B cells in mice and humans is genetically linked to the first tolerance checkpoint.^{75,76} In the absence of AID, autoreactive immature/transitional-1 (T1) B cells are inefficiently purged and exhibit increased resistance to receptor-induced apoptosis.⁷⁵

These substantial effects were surprising in that AID expression in the immature/T1 B-cell pools is only 3% of germinal center (GC) B cells.^{75,77} Noting that Meffre and colleagues had found that equally unlikely candidate for the first tolerance checkpoint in signaling components of TLRs, MyD88, and IRAK4,⁷⁸ we have demonstrated that B-cell antigen receptor (BCR) and Toll-like receptor (TLR) signaling synergize to elicit high levels of AID expression (>50% of GC B cells) in immature/T1 B (but not mature B) cells within 24 hours

of activation (submitted). This synergy is restricted to intracellular TLR ligands and requires both *Myd88* and *Btk*. This synergy pathway mediates an early tolerance checkpoint that appears to be conserved in mice and humans (Figure 2).

Consistent with the requirement of AID and MyD88 for central B-cell tolerance and knowing that intracellular acidification is a necessary step in activating intracellular TLR signaling, we have treated 2F5 KI mice with a week-long regimen of hydroxychloroquine and observed evidence for relaxed B-cell tolerance as increased numbers of developing and mature phenotype B cells (Figure 2) (submitted). These findings identify a novel mechanism for central B-cell tolerance and offer a possible method for increasing the size of bNAb B cell populations available for expansion in response to vaccination.

4.2 | Peripheral control of bNAb responses

Following the lead of checkpoint inhibitors that have proven useful in cancer therapy, we have begun to combine HIV-1 vaccine immunogens with treatment regimens that include antibodies to CTLA-4, PD-1, and CD25. The checkpoint inhibitors, anti-CTLA-4 and PD-1, are being used to maximize helper T-cell activity, first in T-cell zones to promote early B-cell recruitment and expansion and later in GCs to increase T_{FH} activity. Administration of anti-CD25 is targeted for the reduction of T_{Reg} and T_{FR_{reg}} cell numbers in anticipation of prolonging vaccine-induced humoral responses. Initial results suggest some promise for these approaches.

5 | CONCLUSIONS

Since the first description of bnAb polyreactivity and autoreactivity, considerable data have been published that the unusual traits of bnAbs (long HCDR3s, high levels of somatic hypermutation, and poly-and auto-reactivity) indeed contribute to their disfavored status via tolerance control mechanisms. Knockin mouse models have been particularly powerful in dissecting the fate of bnAb lineages in bone marrow and in peripheral immune tissues. New data with B-cell lineage immunogen design of sequential immunizations has been able to induce somatic mutations in bnAb lineages (tian and alt cell), but immune checkpoints in the periphery likely prevent most bnAbs from progressing to full bnAb neutralizing potency and breadth. Clearly, selection of the correct sequential Envs for immunization will be critical, as will selection of the optimal form of Env (ie, minimal immunogens, gp120s, trimers, or multimerized Envs). However, the profile of those that make bnAbs (increased T_{FH}, decreased T_{Reg} cells) raises the hypothesis that in addition to the correct immunogens, for the induction of bnAbs, it will also be critical to formulate the vaccine with adjuvants that foster the bnAb CD4 T-cell profile of increased TFH and decreased TReg cell numbers, and to promote the ability of bnAb B cells to remain in the GC so as to enhance the chance of acquiring key point mutations and insertions that are needed for bnAb breadth.

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FIGURE 1.

UBE3A is a shared autoantigen by 4 CD4bs bNAbs. UBE3A was recognized by VRC01, VRC02, CH106, and CH103 with varying strengths in protein array, and ELISA. (A) Representative protein array summary for protein arrays blotted with CD4bs bNAbs VRC01, VRC02, CH106, CH103, or 151K control. Axis values are fluorescence intensity in 151 array (y-axis) or bNAb array (x-axis). Each dot is the average MFI of duplicate proteins. Major diagonal is equal binding by test Ab and 151K. Dashed line indicates 500-fold MFI threshold to define strong autoreactivity. UBE3A proteins circled. (B) CD4bs bNAb (circle), and 151K (triangle) were tested for binding to UBE3A in sandwich ELISA under stringent conditions.⁵ Used with permission

FIGURE 2.

Chloroquine rescues B-cell development in 2F5 knockin mice. 2F5 mice were injected with PBS or chloroquine for 2 weeks and then killed. B-cell frequencies in bone marrow and spleen were then determined by FACS. Chloroquine increased the frequency of immature and T1 B cells in bone marrow, T1 and T2 B cells in spleen (upper row), and in the mature splenic MZ, and MF B cell compartments (lower row)

TABLE 1

B-cell development in broadly neutralizing antibody knock-in mice

bNAb	Specificity	Result	References
2F5 affinity matured bnAb	gp 41 MPER	Bone marrow: approximately 95% B cells deleted Periphery: residual B cells anergic	(4, 6–8, 10)
4E10 affinity matured bnAb	gp41 MPER	Bone marrow: approximately 95% B cells deleted; receptor editing Periphery: residual B cells anergic	(7, 13)
2F5 germline (UCA)	gp41 MPER	Bone marrow: approximately 95% B cells deleted; Periphery: residual B cells anergic Vaccination: UCA B cells activated with minimal affinity maturation	(19)
3BNC60 germline (UCA)	VRC01-class CD4 binding site	Bone marrow: majority of B cells deleted; Periphery: residual B cells anergic Vaccination: B cells activated with minimal affinity maturation	(79,80)
VRC01 germline with affinity matured HCDR3	VRC01-class CD4 binding site	Bone marrow: no deletion Vaccination: GL B cells activated by with minimal affinity maturation	(81)
VRC01 non-rearranged V _H J _H /V _L J _L germline	VRC01 CD4 binding site	Bone marrow: no deletion; periphery: no anergy Vaccination: GL B cells activated by vaccination with sequential Envs; affinity matured to neutralize glycan deleted HIV mutant viruses; maturation block prior to UBE3A crossreactivity	(82)